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Multiple Determinants for Growth of Human Immunodeficiency Virus Type 1 in Monocyte-Macrophages

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Attempts to define the genetic determinants required for efficient growth of human immunodeficiency virus type 1 (HIV-1) in monocyte-macrophages were made by constructing chimeras between two infectious clones of HIV-1 (HXB2 and LW/C), which despite only minor differences in their DNA sequence have striking differences in cell tropism. Although both of them replicate efficiently in peripheral blood mononuclear cells, HXB2 replicates extensively in permanent T cell lines but poorly in primary monocyte macrophages (T cell line tropic); the reverse is true for LW/C (macrophage tropic). The envelope proved to contain the major determinants of macrophage tropism. However, tropism determinants appeared to be scattered along the envelope. In particular, the V3 loop alone appeared to be neither necessary nor sufficient for growth in macrophages. Both *vpr* and *nef* genes appeared to play a less significant role to improve viral replication in macrophages, but only in the presence of the proper envelope sequences. HIV-1 macrophage tropism thus appears to result from the contribution of several different determinants.

T-lymphocytes and monocyte-macrophages are major targets for human immunodeficiency virus type 1 (HIV-1) infection. T-lymphocytes harbor the virus in the peripheral blood compartment and lymph nodes, while monocyte-macrophages represent a major reservoir in these and many other tissues (*1–5*), such as the central nervous system (*6–11*), the infection of which is ultimately responsible for several clinical manifestations, including acquired immunodeficiency syndrome dementia (*8, 12*). HIV-1 isolates are commonly classified into two major groups based on their tropism. One group (monocyte-macrophage tropic viruses) is represented by viruses which grow efficiently in macrophages and peripheral blood lymphocytes (PBL) but are unable to infect permanent T-cell lines (*7*). A second group (T-cell line tropic) includes viruses which are able to grow in PBL and permanent T-cell lines but fail to grow in monocyte-macrophages. The monocyte-macrophage tropic viruses seem to be more prevalent in viral populations in the early phases of infection. During progression to disease, however, the T-cell tropic variants become more predominant (*13, 14*).

The *env* gene of HIV-1 has been defined as a major determinant of cell tropism (*15–18*). An *env* sequence outside the CD4 binding domain and spanning the V3 loop has been identified as a determinant of tropism for macrophages (*17–19*) as well as for microglial cells (*16*), and the V3 loop alone has been reported as necessary and sufficient to confer macrophage tropism (*15*). Specific amino acid substitutions, both within and outside the V3 loop, affecting the overall conformation of gp120, were shown to play a role in virus infectivity, host range, and syncytium-forming ability (*20*). The V1/V2 region was also shown as necessary to attain full macrophage tropism (*21*). In some strains of HIV-1 even a single amino acid substitution in the V1 loop was responsible for a change in viral tropism (*22*). Recently, *nef* has been shown to be a positive factor of viral replication in primary PBL and macrophages (*23*) and required for high titer production and pathogenesis following *in vivo* infection of macaques with simian immunodeficiency virus (SIV) (*24*). It has been suggested that Vpr, a virion-associated regulatory protein (*25*), plays a role in macrophage tropism for HIV-1 and HIV-2 (*26, 27*).

We report here the results of studies with chimeric viruses containing combinations of fragments of envelope gene from macrophage tropic and T-cell line tropic HIV isolates, and we analyze the contributions of different regions of the viral sequence for their ability to grow in macrophages.

The two infectious molecular clones (pHXB2 and pLW/C) were both derived from HIV-1(HTLV-IIIB) and were consequently very similar in nucleotide identity (2% difference). In spite of their close similarities, viruses derived from these clones had reciprocal cellular host ranges. HXB2 grows extremely well in permanent T-cell lines and peripheral blood mononuclear cells (PBMC), but grows poorly and inconsistently in primary monocyte-macrophages. In contrast, LW/C, constructed from the clone LW 12.3 derived from the isolate of a laboratory worker accidentally infected with HIV-1(HTLV-IIIB), replicates extremely well on monocyte-macrophages and PBMC, but not in T-cell lines.

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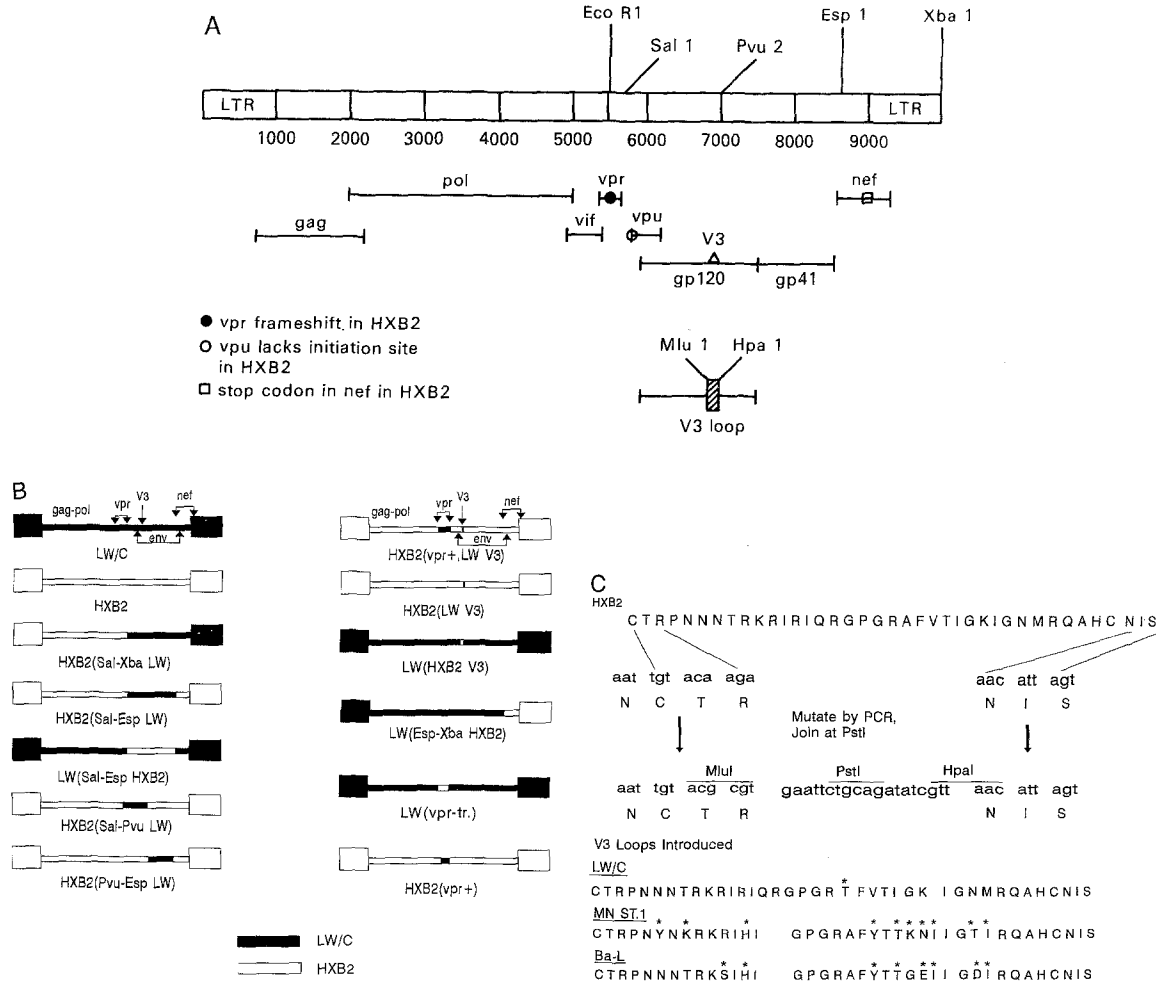

FIG. 1. (A) Genetic structure of molecular clones HXB2 and LW/C and positions of the restriction sites used for chimeric constructs. LW/C clone was prepared from clone LW 12.3 (*33*) by repairing the *vif* and *vpr* genes using the appropriate region from the HIV-1 (HTLV-IIIB) clone pHXB2gpt (*34*). (B) HXB2 (*Sal–Xba* LW) was constructed by replacing the *SalI–XbaI* fragment of HXB2 with the corresponding fragment from LW/C. HXB2 (*Sal–Esp* LW) was constructed by replacing the *SalI–EspI* fragment of HXB2 with the corresponding fragment from LW/C. LW (*Sal–Esp* HXB2) was constructed by replacing the *SalI–EspI* fragment of LW/C with the corresponding fragment from HXB2. HXB2 (*Esp–Xba* LW) was constructed by replacing the *EspI–XbaI* fragment of HXB2 with the corresponding fragment from LW/C. LW (*Esp–Xba* HXB2) was constructed by replacing the *EspI–XbaI* fragment of LW/C with the corresponding fragment from HXB2. HXB2 (vpr^+) was constructed by replacing the *EcoRI–SalI* fragment of HXB2 by the corresponding fragment from LW/C. LW (*vpr*-tr) was constructed by replacing the *EcoRI–SalI* fragment of LW/C with the corresponding fragment from HXB2. HXB2 (*Sal–Pvu* LW) was constructed by replacing the *SalI–PvuII* fragment of HXB2 with the corresponding fragment from LW/C. HXB2 (*Pvu–Esp* LW) was constructed by replacing the *EspI–PvuII* fragment of HXB2 with the corresponding fragment from LW/C. The construction of the V3 loop cassette is as described in Di Marzo Veronese *et al.* (*35*). The IIIB V3 loop sequence, which differs from LW/C by a single amino acid substitution, GPGR**A/T**F, was introduced into LW/C by polymerase chain reaction (PCR)-mediated site-directed mutagenesis. A 0.5-kbp *PvuII–BglII* fragment of LW/C, containing the V3 loop coding region, was amplified in two separate PCRs to generate partially overlapping fragments, each containing the desired mutation. The primers used were: A, 5′-CCGATTCATTAATGCAGCTGAACCA-3′; B, 5′-TTCCTATTGTAACAA-AGGCGCGCCCTGGTCCTCTCTG-3′; C, 5′-CAGAGAGGACCAGGGCGCGCCTTTGTTACAATAGGAA-3′; D, 5′-CACTTCTCCAATTGTCCCTCATATC-3′. Primers A and B were used together, as were primers C and D. Primers B and C introduced a *AscI* site (underlined) in addition to the A to T amino acid substitution. The two fragments were purified, denatured, annealed, and reamplified to produce a mutated *PvuII–BglII* 0.5-kbp fragment, which was substituted for the wild-type LW/C *PvuII–BglII* fragment in an LW/C subclone of a 1.3-kbp *PvuII–BamHI env* subclone in pGEM4 (Promega, Madison, WI). The recombinant *PvuII–BamHI* fragment was substituted for the homologous wild-type fragment in a *SalI–EspI* 2.9-kbp subclone of LW/C in pGEMEX2 (Promega). Finally, the recombinant *SalI–EspI* fragment was substituted for the homologous fragment in the LW/C complete clone in the SP65*gpt* vector, which was used in transfections as a source of infectious virus. The presence of the mutation was verified by *AscI* digestion and by sequencing the amplified mutated region. (C) V3 loop sequences of HXB2, LW/C, MN ST.1, and Ba-L are illustrated. Amino acid residues different from those of HXB2 are indicated by asterisks. Gaps in the sequences are indicated by spaces. The sequence of the V3 loop was from Ratner *et al.* (*36*), that of MN was from Gurgo *et al.* (*37*), and those of LW/C and Ba-L were determined here.

As shown in Fig. 1A, three of the open reading frames (*vpr, vpu,* and *nef*) are complete in LW/C but not in HXB2. The remainder of the proteins from the two viruses, however, are very similar in their inferred amino acid sequences. These infectious clones were accordingly used to construct a variety of chimeric viruses.

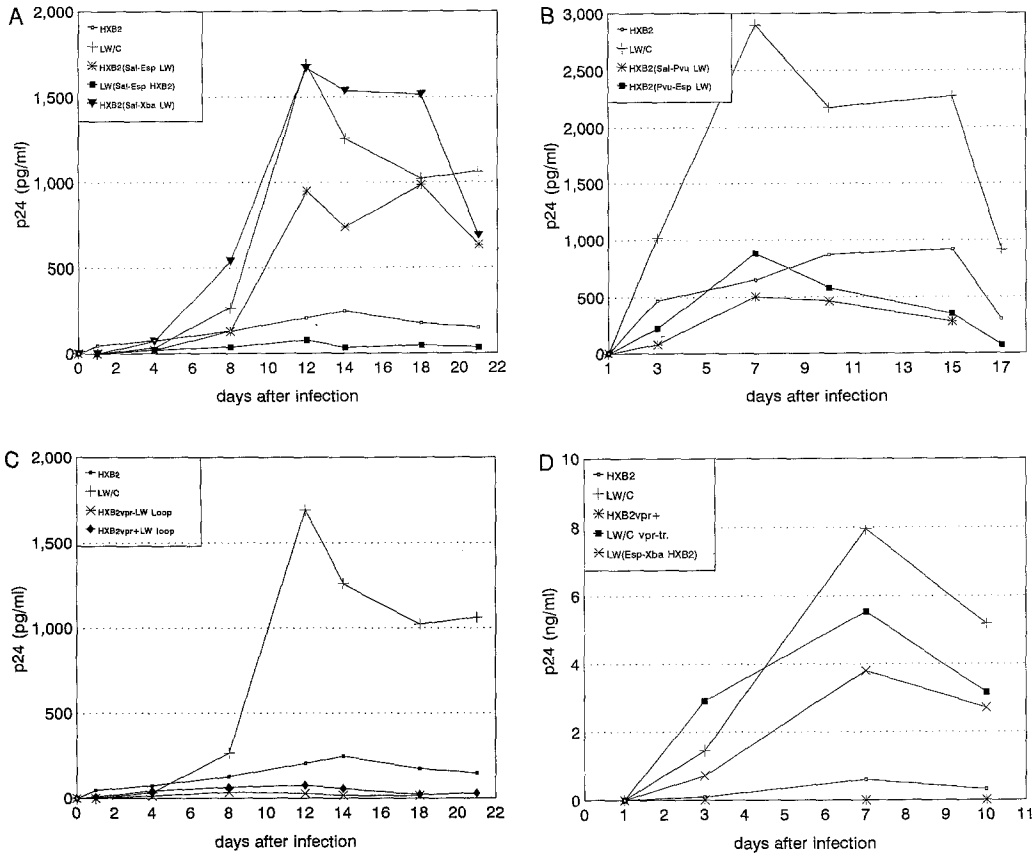

FIG. 2. Growth of HXB2–LW/C chimeric viruses in macrophages. (A) Growth of HXB2, LW/C, HXB2(*Sal–Xba* LW), HXB2(*Sal–Esp* LW), and LW(*Sal–Esp* HXB2). (B) Growth of HXB2, LW/C, HXB2(*Sal–Pvu* LW), and HXB2(*Pvu–Esp* LW). (C) Growth of HXB2 with V3 loop from LW/C. (D) Shown is the kinetic of growth in macrophages of chimeric viruses with substituted *vpr* gene (LW with *vpr* from HXB2 and vice versa). LW(*Esp–Xba* HXB2) contains *nef* from HXB2. Virus preparation: Transfection of HeLa-Tat cells (*38*) with plasmids containing chimeric constructs was performed by using the calcium precipitation method (*39*). Cell culture supernatant was collected after 48 hr and then used as the viral source for the infection of macrophages. Cell preparation: Blood from normal donors was used for the Ficoll gradient centrifugation preparation of PBMC. Monocytes were selected by adherence on the plastic pretreated with human serum. For 24-well plates, 5×10^6 PBMC were plated per well. For 25-cm^2 flask, 5×10^7 PBMC were plated. Macrophages were grown in the presence of 10 U/ml of granulocyte-macrophage colony stimulating factor in RPMI 1640 containing 10% fetal calf serum (Hyclone, Logan, UT) 14 days before infection. Infection and virus detection: Macrophages were infected by using an amount of virus corresponding to 100,000 cpm of reverses transcriptase (RT) activity per flask or 10,000 cpm per well. Production of HIV-1 p24 was measured by antigen capture assay. RT activity was measured by standard protocols. Results shown are representatives of at least three experiments on macrophages derived from different blood donors.

The ability of different viral chimerae to grow in macrophages indicated the importance for macrophage tropism of determinants in the LW/C *env* gene (Figs. 2A and 2B). HXB2(*Sal–Xba* LW), which contained the right half (including *vpu, env, nef, tat, rev,* and 3′ long terminal repeat) of the LW/C provirus, replicated in macrophages nearly as efficiently as LW/C (Fig. 2A). Another pair of reciprocal chimeras contained either the *vpu, rev, tat,* and *env* of LW within the HXB2 genetic backbone (HXB2[*Sal–Esp* LW]) or vice versa (LW[*Sal–Esp* HXB2]). Substitution of the *Sal*I–*Esp*I fragment of HXB2 into LW/C resulted in a virus with a low replication rate in macrophages. In contrast, the *Sal*I–*Esp*I fragment of LW/C conferred on HXB2 the ability to replicate in macrophages at 55–60% the rate of LW/C (Fig. 2A).

The *env* gene of the IIIB family of HIV-1 contains a *Pvu*II site just 5′ of the coding region for hypervariable region 3 (V3), a loop bounded by cysteine residues which constitutes a target for neutralizing antibodies and which has been reported to be critical for cellular host ranges. Substitution of the *Sal*I–*Pvu*II or the *Pvu*II–*Esp*I fragment from LW/C into HXB2 did not significantly increase the ability of the resultant viruses to grow in macrophages (Fig. 2B). These data suggested that determinants distributed over different parts of the *env* gene of LW/C contribute in concert to its ability to grow in macrophages, but are not active individually. They also suggested that the V3 of LW/C did not play a primary role in determining its ability to grow on macrophages.

To further ascertain the contribution of the V3 region to the determination of the ability of LW/C but not HXB2 to grow on macrophages, a series of chimeric viruses were constructed in which only the V3 loops were changed. HXB2 containing the V3 loop of LW/C grew as

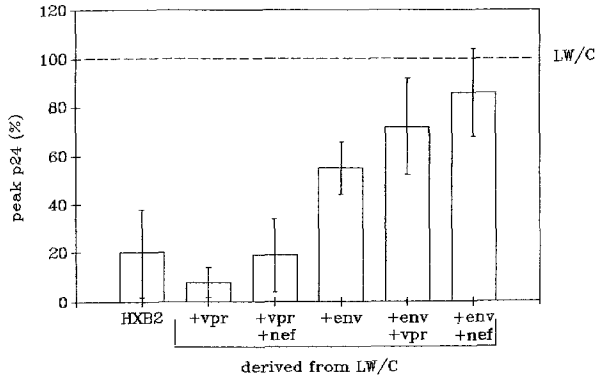

FIG. 3. Contribution of *env, vpr,* and *nef* for growth in macrophages. Calculation of the average growth in macrophages (compared to the LW/C clone) of clones which had LW/C genes in the backbone of HXB2. (*X* axis legend) HXB2, average for HXB2 and HXB2(LWV3); *+vpr*, average for HXB2*vpr*+; *+vpr + nef*, average for LW(*Sal–Esp* HXB2); +env, average for HXB2(*Sal–Esp* LW); +env + vpr, average for LW(*Esp–Xba* HXB2); *+env + nef*, average for LW(*Sal–Xba* HXB2); and growth of LW/C is shown as 100%.

poorly in macrophages as did HXB2 (Fig. 2C). A chimera of LW/C which contained the V3 loop of HXB2 replicated as well in macrophages as did the parental LW/C (not shown).

Chimeras were also constructed which contained the V3 loops of two other macrophage tropic viruses, HIV-1(Ba-L) and (MN ST.1). Neither of the HXB2 chimeric viruses containing V3 loops from MN ST.1 or Ba-L replicated in macrophages more efficiently than HXB2 itself (not shown).

LW(*Esp–Xba* HXB2), which has the incomplete *nef* of HXB2, showed a decrease in the ability to replicate in macrophages when compared with LW/C (Fig. 2D). Furthermore, HXB2(*Sal*I–*Xba*I LW), containing both the *env* and *nef* genes of LW, grew better in macrophages than did HXB2(*Sal*I–*Esp*I LW), which has the truncated *nef* gene of HXB2 (Fig. 2A). However, LW(*Sal–Esp* HXB2), containing the *nef* gene of LW but the *env* gene of HXB2 could not grow in macrophages (Fig. 2B). These data suggested that *nef* was able to positively contribute in growth in macrophages, but only in the context of viruses with an effective (i.e., LW/C) envelope.

Figure 3 illustrates data obtained by calculating the average growth in macrophages for at least three experiments of the clones which had either *vpr, nef,* or *env* or the combination of these genes from LW/C, in the backbone of HXB2. Such analysis confirmed that *env* is the major determinant for macrophage tropism. Furthermore, both *vpr* and *nef* contributed to better growth in macrophages, but to a lesser extent, and only when the macrophage-tropic envelope was also present. Although the difference between averages of HXB2(*Sal–Esp* LW) (*+env*) and LW(*Sal–Xba* HXB2) (*+env + nef*) was not statistically significant (only 27%), and the difference between *env*+ and *env + vpr* was 17%, one can claim the existence of tendency for positive contribution of *vpr* and *nef* genes for growth of HIV-1 in macrophages.

The appropriate *env* gene of HIV-1 has been reported to be necessary and sufficient to confer tropism for macrophages upon HIV-1 (*11, 15–17*). Our data are at least partially consistent with this idea, since the macrophage tropism of our constructs correlates with the presence of the *env* gene of the macrophage tropic clone LW/C (see Fig. 2A). However, our data showed that the determinants of the envelope gene necessary for the macrophage tropism of LW/C did not map to a single region, since two subclones containing only part of the LW/C *env* sequence (the first one spanning the region of V1 and V2 loops and the second one spanning the region of V3 loop, as well as CD4 binding site and gp41) failed to grow as efficiently in macrophages as LW/C (see Fig. 2B). This is in agreement with recent observations with SIV showing that the determinants for macrophage tropism are scattered along the *env* gene (*28*) and study of the role of V1/V2 domains in cell tropism (*21*).

A restricted region of the envelope, the V3 loop, which is known to be the principal neutralizing determinant of HIV-1 (*29–31*), has been reported to be sufficient for macrophage tropism (*15*). Our data do not support this conclusion, since all our constructs containing the V3 loop from viruses able to grow well in monocyte-macrophages within the envelope from a nonmacrophage tropic virus, HXB2, show little or no growth in macrophages. Moreover, LW/C containing the V3 loop of the HXB2 clone was still able to grow in macrophages at levels comparable with the LW/C wild-type, indicating that V3 loop was not only insufficient but also unnecessary to determine the macrophage tropism of LW/C. These results, taken as a whole, suggest that the properties of the envelope to confer the ability of the virus to infect and be expressed in macrophages are determined by the overall tertiary structure of the envelope protein rather than by particular limited stretches of contiguous amino acid residues. In some viral strains the V3 loop could be the primary determinant (*15*). In others, like those analyzed here, the determinants necessary to confer macrophage tropism upon the nonmacrophage tropic partner are more extensively distributed and a larger sequence must be switched for efficient growth.

In our experiments, repair of *vpr* in HXB2 did not confer the ability to replicate in macrophages unless in the context of the proper *env*. Since it has been suggested (*32*) that *vpr* is necessary for efficient replication of the virus in $CD4^+$ cell cultures, *vpr* may have a generic effect on the efficacy of HIV-1 replication rather than its specific role in macrophage tropism. Similar consideration could apply to the role of *nef* (*23*). Although the role of *vpu* has not been addressed in these experiments, indications that *vpu* does not play a specific role on macrophage tropism have been obtained by using another clone, MN-ST1 (*33*) (not shown).

In conclusion, we suggest that macrophage tropism of HIV-1 does not seem to have a single discrete determinant. The V3 loop alone appears to be neither necessary nor sufficient to confer macrophage tropism on HXB2 or reduce it with LW/C. Reports from others (*15*), which indicate that the V3 loop is both necessary and sufficient to confer macrophage tropism, could be due to the different genetic context (HXB3 versus HXB2) into which their chimeric loops have been introduced. The assignment of tropism determinants thus may depend on the particular pair of viruses being compared. Our results indicate that determinants from macrophage-tropic viruses not contained in V3 are required for the ability to grow in macrophages. However, while the proper tertiary structure of envelope appears to be critical for growth in macrophages, regions outside the envelope (such as *nef* containing region downstream from the *env* and *vpr*) could also be important, but only when the proper envelope gene is present.

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